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(54) Title: HIGH THROUGHPUT SCREEN FOR INHIBITORS OF FATTY ACID BIOSYNTHESIS IN BACTERIA

(57) Abstract: Methods for identifying compounds that are inhibitors of bacterial fatty acid biosynthesis are disclosed. Such compounds can be used as lead compounds in methods for preparing antibacterial agents for treating bacterial infections (e.g., in humans, animals, and plants). Inhibitors of bacterial fatty acid synthesis can also be tested for their ability to inhibit synthesis of acylated homoserine lactones. Compounds that inhibit synthesis of acylated homoserine lactones can be used as inhibitors of bacterial virulence. The disclosed methods allow for high throughput screening of libraries of test compounds.

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## HIGH THROUGHPUT SCREEN FOR INHIBITORS OF FATTY ACID BIOSYNTHESIS IN BACTERIA

### FIELD OF THE INVENTION

5 The invention relates to methods for identifying inhibitors of fatty acid biosynthesis in bacteria.

### BACKGROUND OF THE INVENTION

Fatty acid biosynthesis (FAB) is necessary for the production of bacterial cell walls, and therefore is essential for the survival of bacteria (Magnuson et al., 1993, Microbiol. Rev. 57:522-542). The fatty acid synthase system in *E. coli* is the archetypal type II fatty acid synthase system. Multiple enzymes are involved in fatty acid biosynthesis, and genes encoding the enzymes *fabH*, *fabD*, *fabG*, *acpP*, and *fabF* are clustered together on the *E. coli* chromosome. Clusters of FAB genes have also been found in *Bacillus subtilis*, *Haemophilus influenza* Rd, *Vibrio harveyi*, and *Rhodobacter capsulatus*. Examples of FAB genes in *B. subtilis* include *fabD*, *yjaX*, and *yhfB* (encoding synthase III), *fabG*, *ywpB*, *yjhW*, *yjaY*, *ylpC*, *fabG*, and *acpA*. The *ylpC*, *fabG*, and *acpA* genes are contained within a single operon that is controlled by the *PylpC* promoter.

Using genetic and biochemical methods, the targets and mechanisms of action of several inhibitors of the elongation part of the FAB pathway have been identified (Fig. 1). The FAB inhibitor cerulenin inhibits  $\beta$ -ketoacyl-ACP synthase, and the inhibitor thiolactomycin inhibits acetoacyl ACP synthase (Omura, 1981, Meth. Enzymol. 72:520-532; Moche et al., 1999, J. Biol. Chem. 274:6031-6034; and Jackowski et al., 1989, J. Biol. Chem. 264:7624-7629). The FAB inhibitors isoniazid, triclosan, and diazaborine inhibit enoyl-acyl carrier protein reductase (Quemard et al., 1995, Biochem. 34:8235-8241; McMurray et al., 1998, Nature 394:531-532; Heath et al., 1998, J. Biol. Chem. 273:30316-30320; Heath et al., 1999, J. Biol. Chem. 274:11110-11114; Levy et al., 1999, Nature 398:383-384; and DeBoer et al., 1999, Mol. Microbiol. 31:443-450). In *Pseudomonas aeruginosa*, triclosan has been shown to inhibit the enoyl-acyl carrier protein reductase (FabI), which converts *trans*-2-enoyl-ACP to acyl-ACP (Hoang and Schweizer, *supra*). The FAB pathway provides the acyl

groups for production of acylated homoserine lactones (HSLs). HSLs are the signaling molecules involved in quorum sensing, i.e., bacterial cell-to-cell signaling, in a wide variety of bacteria. In pathogenic bacteria, such as *Pseudomonas*, quorum sensing is a mechanism for regulating the expression of virulence factors (Hastings and Greenberg, 1999, J. Bacteriol. 181:2667-2668).

## SUMMARY OF THE INVENTION

The invention is based upon the discovery that the activity of promoters of certain genes is increased in the presence of compounds that inhibit *B. subtilis* FAB. Thus, compounds that inhibit FAB can be identified by their ability to increase the activity of the *B. subtilis* PyhfB and PylpC promoters. Various promoters can be used in the invention, provided that the activity of the promoter is upregulated by a FAB inhibitor, such as cerulenin or triclosan. FAB inhibitors that slow the growth of, or kill, bacteria are candidate antibacterial agents that can be used in methods of treating bacterial infections. The invention thus provides a rapid and convenient method for identifying (i) compounds that inhibit FAB and which can subsequently be derivatized to produce antibacterial agents, as well as (ii) compounds that inhibit FAB and which are antibacterial agents. Because the FAB pathway is involved in the synthesis of HSLs, the invention also provides a method for identifying compounds that inhibit HSL synthesis. Such compounds can be used to inhibit bacterial virulence. If desired, such inhibitors of HSL synthesis can be further derivatized using standard medicinal chemistry techniques to produce inhibitors of virulence having increased potency.

Accordingly, the invention features a method for determining whether a test compound is an inhibitor of bacterial FAB. The method includes: (i) contacting a bacterial cell with a test compound, wherein the bacterial cell contains (a) a promoter (e.g., PyhfB or PylpC), the activity of which is increased in the presence of a compound that inhibits FAB, operably linked to (b) a reporter gene; and (ii) measuring activity of the promoter, wherein an increase in activity, relative to the level of activity of the promoter in the absence of the test compound, indicates that the test compound is an inhibitor of bacterial FAB.

The invention also includes a method for determining whether a test compound is an antibacterial agent, the method comprising: (i) contacting a bacterial cell with a test

compound, wherein the bacterial cell contains (a) a promoter (e.g., *PyhfB* or *PylpC*), the activity of which is increased in the presence of a compound that inhibits FAB, operably linked to (b) a reporter gene; (ii) measuring activity of the promoter, wherein an increase in activity, relative to the level of activity of the promoter in the absence of the test compound, indicates that the test compound is an inhibitor of FAB; and (iii) determining whether the compound is an antibacterial agent by determining whether the compound kills, or slows the growth of, bacteria. Optionally, the test compound may be further assayed in a biochemical assay (e.g., in an extract of the cell) to determine which step in the pathway is inhibited, and to confirm that the test compound inhibits fatty acid biosynthesis. For example, inhibition of fatty acid biosynthesis can be detected as inhibition of incorporation of acetate into fatty acids or phospholipids. Conventional methods can be used to measure inhibition of incorporation of acetate.

An increase in activity of the promoter can be measured, for example, by measuring expression of a reporter gene that is operably linked to the promoter, such as a *lacZ*, *cat*, *gus*, a green fluorescent protein gene, or a luciferase gene. Other suitable reporter genes are well known in the art and can be used in the invention. If desired, the activity of the promoter can be measured by measuring binding of antibodies to a product of the reporter gene (e.g., a protein encoded by the reporter gene), with an increase in the level of bound antibodies reflecting an increase in activity of the promoter. Alternatively, activity can be measured by measuring the level of mRNA transcribed from the reporter gene, with an increase in the mRNA level reflecting an increase in promoter activity.

The invention also provides methods of preparing (i) an inhibitor of fatty acid biosynthesis and/or (ii) an antibacterial agent. The methods include: screening multiple test compounds by the methods described above; identifying candidate compounds that upregulate promoter activity; isolating one or more lead compounds from the candidate compounds; identifying and selecting a lead compound that inhibits fatty acid biosynthesis or bacterial growth; and formulating the selected lead compound as an inhibitor of fatty acid biosynthesis or as an antibacterial agent. A "lead compound" is a test compound that increases promoter activity by at least 3 times the standard deviation, plus the mean. If desired, lead compounds can subsequently be derivatized using conventional medicinal chemistry methods, as described herein.

Similarly, the invention features methods for preparing (i) an inhibitor of FAB or (ii) an antibacterial agent. The methods include screening multiple test compounds by the methods described above: identifying candidate compounds that upregulate promoter activity; isolating one or more lead compounds from the candidate compounds; derivatizing the lead compound(s), thereby producing a derivative of the lead compound; identifying derivatives that inhibit FAB or bacterial growth; and formulating the derivative as an inhibitor of FAB or as an antibacterial agent (e.g., by admixture with a pharmaceutically acceptable carrier). Inhibitors of FAB and antibacterial agents prepared by such methods also are included within the invention. Such compounds can be used in methods for inhibiting bacterial fatty acid biosynthesis or growth of bacteria in an organism having a bacterial infection.

The invention also provides a method for identifying an inhibitor of bacterial virulence. In such a method, a FAB inhibitor identified by the methods disclosed herein can be further tested for its ability to inhibit the synthesis of HSL. Inhibition of acylated homoserine lactone synthesis can be detected as inhibition of enoyl-acyl carrier protein reductase activity. In a convenient assay, the FAB inhibitor can be tested *in vitro* for its ability to inhibit the synthesis of *N*-butyryl-L-homoserine lactone (C<sub>4</sub>-HSL) in the presence of FabI (enoyl-acyl carrier protein reductase), the HSL synthetase RhII, the cofactor NADH, and the substrates crotonyl-ACP and S-adenosylmethionine (see, e.g., Hoang and Schweizer, *supra*). A decreased level of HSL synthesis in the presence of the test compound, relative to the level of HSL synthesis in the absence of the test compound, indicates that the FAB inhibitor is an inhibitor of HSL synthesis. Because HSLs regulate the expression of virulence factors, inhibitors of HSL synthesis can be used to inhibit bacterial virulence, e.g., in a method for treating a bacterial infection. If desired, such HSL synthesis inhibitors can be derivatized, using standard medicinal chemistry techniques as described herein, to produce derivatives that have increased potency as inhibitors of virulence in bacteria.

The invention offers several advantages. For example, various embodiments of the invention can readily be used for high-throughput screening (HTS) of a wide variety of test compounds. Thus, lead compounds can readily be selected from a large number of test compounds. Assays employing the Pyh1B and PylpC promoters are capable of detecting FAB inhibitors at concentrations both above and below their minimal inhibitory concentration (MIC). Thus, the assays described herein provide a high level of sensitivity

and are expected to detect growth inhibitory (i.e., bacteriocidal) compounds, as well as less potent inhibitors of the FAB pathway, which can be subsequently modified using standard medicinal chemistry techniques and by evaluating structure-activity relationship (SAR) data. Because the assays are cell-based, the assays identify antibacterial agents that can efficiently enter bacterial cells. Thus, the assays allow the identification of potent antibacterial compounds and compounds of structural interest that may have relatively modest potency, but have favorable cell permeability properties. In addition, because FAB is a biochemical pathway, the methods disclosed herein enable the identification of compounds that may inhibit any enzymatic function or step in the pathway.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, technical manuals, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of the bacterial fatty acid biosynthesis pathway. The targets of several fatty acid biosynthesis inhibitors are shown.

Fig. 2 is a listing of the nucleotide sequence of the PyhfB promoter.

Fig. 3 is a listing of the nucleotide sequence of the PyjaXY promoter.

Fig. 4 is a listing of the nucleotide sequence of the PylpC promoter.

Fig. 5 is a listing of the nucleotide sequence of the PyydK promoter.

Fig. 6 is a map of the pDG268 vector.

Fig. 7 is a histogram depicting the level of induction of the PyhfB promoter in the presence of varying concentrations of triclosan (tri) and cerulenin (cer).

Fig. 8 is a graph depicting the level of induction of the PylpC promoter in the presence of varying concentrations of triclosan (tri) and cerulenin (cer).

### DETAILED DESCRIPTION

The invention provides a method for determining whether a test compound is an inhibitor of bacterial FAB. The invention derives from the discovery that compounds that inhibit fatty acid biosynthesis cause an increase in the activity of the PyhfB and PylpC promoters. Generally, the method involves determining whether the test compound induces an increase in activity in the PyhfB or PylpC promoter, as indicated by an increase in expression of a reporter gene operably linked to the promoter (i.e., a reporter-based screen). As discussed in detail below, the activity of the PyhfB and PylpC promoters is increased upon inhibition of the FAB pathway. Thus, compounds that increase the activity of the PyhfB or PylpC promoter can be expected to inhibit bacterial fatty acid biosynthesis. Optionally, the test compound can be further tested to confirm that it inhibits fatty acid biosynthesis (i.e., in a biochemical screen). These methods are described in further detail below.

#### Part I: Reporter-Based Screens

Genetic Constructs: Conventional transcriptional profiling methods can be used to identify promoters that have increased activity in the presence of antibacterial agents such as cerulenin and triclosan, which are inhibitors of FAB. In such an assay, certain bacterial promoters display an increase in activity in the presence of antibacterial agents, as evidenced by an increase in the level of mRNA transcripts of the sequences.

A "promoter" is a minimal sequence sufficient to direct transcription; the promoter is located in the 5' region of the native gene. A sequence containing the PyhfB promoter is set forth in Fig. 2 as SEQ ID NO:1. The sequence of the *yhfB* gene is set forth in GenBank as Accession No. BG13048. A sequence containing the PyjaXY promoter is set forth in Fig. 3 as SEQ ID NO:2. The sequence of the *yjaX* gene is set forth in GenBank as Accession No. BG13127. A sequence containing the PylpC promoter is set forth in Fig. 4 as SEQ ID NO:3. The sequence of the *ylpC* gene is set forth in GenBank as Accession No. BG13400. A

sequence containing the PyydK promoter is set forth in Fig. 5 as SEQ ID NO:4. The sequence of the *yydK* gene is set forth in GenBank as Accession No. BG11484.

To confirm that the activity of a promoter is increased in the presence of an antibacterial agent, each promoter can be operably linked to the coding sequence of a reporter gene, such as the *E. coli lacZ* gene. The resulting genetic constructs then are inserted into a silent chromosomal location in *B. subtilis*. Such strains are treated with an antibacterial agent, such as cerulenin or triclosan, and the level of promoter activity, as measured by *lacZ* expression is measured. An increase in the level of promoter activity, relative to the level of *lacZ* expression in untreated, control cells, confirms that promoter activity is modulated by the antibacterial agent.

The "reporter gene" can be any sequence the expression of which can be detected or measured, other than the coding sequence to which the promoter naturally is operably linked. Typically, the reporter gene is heterologous to the bacterial strain in which promoter activity is measured. Examples of suitable reporter genes include, without limitation, *lacZ*, the bacterial chloramphenicol transacetylase (*cat*) gene, luciferase genes, the bacterial *gus* gene, and the like. Also included are sequences that encode fluorescent markers, such as green fluorescent protein (GFP). The aforementioned reporter genes, and methods for measuring their expression, are well known in the art.

Bacterial Strains: *B. subtilis* strains having the PyhfB or PylpC promoter operably linked to the coding sequence of a reporter gene can be used to assay the ability of test compounds to increase activity of the PyhfB or PylpC promoters. If desired, the promoter and reporter gene can be stably integrated into the bacterial chromosome. Examples of such strains include PY79 (*amy::PyhfB-lacZ, cat*) and PY79 (*amy::PylpC-lacZ, cat*).

Alternatively, the promoter and coding sequence of the reporter gene can be located on a plasmid that is introduced into a bacterium (e.g., *E. coli*). If desired, a test compound can first be identified as an antibacterial agent by detecting its ability to increase the activity of one of the aforementioned *B. subtilis* promoters, and then confirming its ability to act as an antibacterial agent by detecting its ability to increase the activity of a second promoter. For example, test compounds can first be screened with the strain PY79 (*amy::PyhfB-lacZ, cat*). Test compounds that increase the activity of the PyhfB promoter can then be tested for their ability to increase the activity of the PylpC promoter in the strain PY79 (*amy::PylpC-lacZ, cat*).



Test Compounds: The "test compound" can be any compound, such as a small organic molecule, amino acid, polypeptide, nucleic acid, peptide nucleic acid, carbohydrate, or polysaccharide. The test compound can be synthetic, naturally occurring, or a combination of synthetic and natural components. If desired, the test compound can be a member of a library of test compounds (e.g., a combinatorial chemical library) or a component of a cellular extract or bodily fluid (e.g., urine, blood, tears, sweat, or saliva).

Test compounds that increase the activity of the promoter, relative to the level of promoter activity in the absence of the test compound, are considered inhibitors of bacterial FAB. The level of promoter activity measured in the presence of the test compound then is compared with the level measured in the absence of the test compound. Generally, an increase in the level of promoter activity that is at least 3 times the standard deviation of a test compound set (i.e., a set of one or more test compounds), plus the mean of a test compound set, relative to the level of gene expression in the absence of the test compound, indicates that the test compound is an inhibitor of FAB. Such an increase may be, for example, 5-fold, 10-fold, 20-fold, 50-fold, or even 100-fold. A relatively high level of induction generally indicates that the test compound has a relatively high level of potency.

Promoter activity, as measured by reporter gene expression, can be measured by any of a number of conventional methods, and the optimal method will depend upon factors such as the nature and function of the reporter gene. In general, suitable assays of reporter gene expression include methods such as (i) assaying the function of a product of the reporter gene (e.g., measuring an enzymatic reaction catalyzed by a product of the reporter gene); (ii) measuring the level of protein expressed from the reporter gene (e.g., by SDS-PAGE or in an immunoassay using antibodies (e.g., polyclonal or monoclonal antibodies) that specifically bind to the product of the reporter gene; and (iii) measuring the level of mRNA transcribed from the reporter gene. Included within the invention are assays that permit high throughput screening of test compounds.

The assays of promoter induction can be carried out in virtually any reaction vessel or receptacle. Examples of suitable receptacles include 96-well plates, 384-well plates, test tubes, centrifuge tubes, and microcentrifuge tubes. The methods can also be carried out on surfaces such as metal, glass, ceramics, paper, polymeric chips, membrane surfaces, resins, or the surface of a matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS) plate.

Part II: Biochemical Screen

Once a test compound is identified as an inhibitor of FAB using the above-described reporter-based assay, the compound can be further tested, if desired, in a biochemical assay. Such a biochemical assay generally involves measuring inhibition of fatty acid biosynthesis. Various methods for measuring inhibition of fatty acid biosynthesis are known in the art, and can be used in this aspect of the invention (see, e.g., Buttke et al., Biochemistry 17:5282-5286, 1978; Kuhajda et al., PNAS 91:6379-6383, 1994; Weiss et al., Biol. Chem. Hoppe Seyler 367:905-912, 1986; Stoops et al., J. Biol. Chem. 253:4464-4475, 1978; Duronio et al., J. Cell Biol. 113:1313-1330, 1991; Meyer et al., 117:345-350, 1974; Zhao et al., Microbiol. 142:2509-2514, 1996; and Schneiter et al., Mol. Cell. Biol. 16:7161-7172, 1996, which are incorporated herein by reference). Table 1 sets forth various *B. subtilis* and *E. coli* fatty acid biosynthesis genes and enzymes, along with the enzymatic reactions that the enzymes catalyze. Standard assays for measuring inhibition of the enzymatic reactions are summarized in Table 1 and can be used in the invention.

If desired, inhibition of fatty acid biosynthesis can be measured in a cell extract that contains all of the components (e.g., enzymes, cofactors, carrier molecules, and buffers) normally necessary for a particular step that is needed in the synthesis of fatty acids. Cell extracts containing enzymes, cofactors, and carrier molecules can be cytoplasmic, cytosolic, or membrane preparations, whole cells, or naturally-occurring or synthetic mixtures composed of natural or unnatural components, or both. Carrier molecules included in the cell extract can include numerous components, such as molecular transport machinery and membranes. The substrate for the reaction can be contained within the cell extract initially, it can be added in solution, e.g., as a dry or liquid additive, or it can be generated *in situ* (e.g., as the product of another reaction). The substrate can be detectably labeled with a tag, for example, a radiolabel, a fluorescent label, a magnetic label, or as a biotinylated derivative.

Table 1: Assays of Fatty Acid Biosynthesis Enzymes

| <i>B. subtilis</i> Gene and Enzyme          | <i>E. coli</i> Gene and Enzyme              | Reaction Catalyzed by the Enzyme                                      | Assay  |
|---|---|---|--|
| <i>fabD</i><br>malonyl CoA-ACP transacylase | <i>fabD</i><br>malonyl CoA-ACP transacylase | malonyl-CoA + ACP → malonyl-ACP + CoA                                 | measure incorporation of <sup>14</sup> C malonate into ACP                 |
| <i>yjaX</i> and <i>YhfB</i><br>synthase III | <i>fabH</i><br>acetoacyl-ACP synthase       | malonyl-ACP + acetyl-CoA → acetoacyl-ACP + CO <sub>2</sub>            | measure incorporation of <sup>14</sup> C acetyl-CoA into ACP               |
| <i>fabG</i><br>β-ketoacyl-ACP reductase     | <i>fabG</i><br>β-ketoacyl-ACP reductase     | acetoacyl-ACP + NADPH → D-hydroxybutyryl-ACP + NADP                   | measure NADPH oxidation at 340 nm  |
| <i>ywpB</i><br>β-hydroxyacyl-ACP dehydrase  | <i>fabA</i><br>β-hydroxyacyl-ACP dehydrase  | β-hydroxybutyryl-ACP ⇌ Crotonyl-ACP + H <sub>2</sub> O                | in conjunction with <i>fabI</i> , measure NADH oxidation                   |
| <i>yjbW</i><br>enoyl reductase              | <i>fabI</i><br>enoyl reductase              | crotonyl-ACP + NADH → butyryl-ACP + NAD                               | measure NADH oxidation at 340 nm   |
| <i>yjaY</i><br>β-ketoacyl ACP synthase      | <i>fabF/B</i><br>β-ketoacyl ACP synthase    | fatty acyl-ACP + malonyl-ACP → β-ketoacyl-ACP + CO <sub>2</sub> + ACP | measure β-ketoacyl formation at 303 nm in the presence of Mg <sup>2+</sup> |

After incubation of the cell extract/substrate mixture under conditions that normally allow the particular step(s) to proceed, the mixture is assayed to determine whether the substrate remains and/or whether the corresponding product or products have been formed. The optimal duration of incubation varies with the particular synthesis step(s) being carried out and also with incubation temperature (e.g., at least 1 hour, 12 hours, 1 day, 2 days, a week, or longer, at, e.g., room temperature or lower, 30°C, 37°C, or higher, depending on the strain of bacteria).

#### Medicinal Chemistry

Once a compound has been identified as an inhibitor of FAB, principles of standard medicinal chemistry can be used to produce derivatives of the compound. The moieties that are responsible for the compound's activity can be revealed by examining its structure-activity relationships (SAR). Specifically, a person of ordinary skill in the art of chemistry could modify a moiety of the compound to study the effects of the modification on the potency of the compound and thereby produce derivatives of the compound having increased potency (See, e.g., Nagarajan et al., Antibiot. 41:430-438). For example, chemical modifications such as N-acylation, esterification, hydroxylation, alkylation, amination, amidation, oxidation, or reduction can be made. Such chemical modifications can be made according to conventional methods (See, e.g., Wade, Organic Chemistry, Prentice-Hall, Inc.,

New Jersey, 1987). If structural information of the FAB inhibitor and its target are available, derivatives of the inhibitor can be generated and optimized virtually by using molecular modeling software and conventional methods. Such software is commercially available (e.g., from Tripos Inc., Molecular Simulations, Inc., and MDL Information Systems, Inc).

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#### Use of FAB Inhibitors

A compound identified as a FAB inhibitor or as an antibacterial agent can be used to treat a bacterial infection in an organism (e.g., a plant or mammal, such as a human, dog, cat, cow, etc.). Because fatty acid biosynthesis genes are widely conserved, inhibitors of fatty acid biosynthesis are expected to be useful in inhibiting fatty acid biosynthesis in a wide spectrum of bacteria. For example, the compounds can be used to treat infections with gram positive bacteria (e.g., *Bacillus* spp.), particularly pathogenic bacteria, such as *Streptococcus* spp. Thus, the compounds can be used, for example, to treat infections caused by *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus endocarditis*, *Streptococcus faecium*, *Streptococcus sanguis*, *Streptococcus viridans*, or *Streptococcus hemolyticus*. The compounds that are identified as FAB inhibitors or as antibacterial agents using the screening methods of the invention are expected to have activity against a broad spectrum of bacteria.

A composition containing an effective amount of a FAB inhibitor or an antibacterial agent can be administered to an organism in a method of treatment (e.g., topically, orally, nasally, buccally, subcutaneously, or intraperitoneally). Treatment typically includes administering an effective amount of a composition containing a FAB inhibitor or an antibacterial agent to a subject in need of such treatment, thereby inhibiting bacterial growth in the subject. Such a composition typically contains from about 0.1 to 90% by weight (preferably 1 to 20% or 1 to 10%) of the antibacterial agent of the invention in a pharmaceutically acceptable carrier.

Solid formulations of the compositions for oral administration may contain suitable carriers or excipients such gelatin, lactose, acacia, sucrose, kaolin, mannitol, dicalcium phosphate, calcium carbonate, sodium chloride, or alginic acid. Disintegrators that can be used include, without limitation, micro-crystalline cellulose, corn starch, sodium starch glycolate and alginic acid. Tablet binders that may be used include acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone (Povidone™), hydroxypropyl

methylcellulose, sucrose, starch, and ethylcellulose. Lubricants that may be used include magnesium stearates, stearic acid, silicone fluid, talc, waxes, oils, and colloidal silica.

Liquid formulations of the compositions for oral administration prepared in water or other aqueous vehicles may contain various suspending agents such as methylcellulose, alginates, tragacanth, pectin, kelgin, carrageenan, acacia, polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations may also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents. Various liquid and powder formulations can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

Injectable formulations of the compositions may contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). For intravenous injections, water soluble versions of the compounds may be administered by the drip method, whereby a composition containing the compound and a physiologically acceptable excipient is infused. Physiologically acceptable excipients may include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. For intramuscular preparations, a sterile formulation of a suitable soluble salt form of the compounds can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such as an ester of a long chain fatty acid (e.g., ethyl oleate).

A topical semi-solid ointment formulation typically contains a concentration of the active ingredient from about 0.1 to 20% wt/vol (preferably 0.1 to 2% wt/vol of essentially pure material) in a carrier such as a pharmaceutical cream base. Various formulations for topical use include drops, tinctures, lotions, creams, solutions, and ointments containing the active ingredient and various supports and vehicles.

The optimal percentage of the active ingredient in each composition varies according to the formulation itself and the therapeutic effect desired. Appropriate dosages can readily be determined by those of ordinary skill in the art by monitoring the organism for signs of disease amelioration or inhibition, and increasing or decreasing the dosage and/or frequency of treatment as desired. The optimal amount of the composition used for treatment of

conditions caused by or contributed to by bacterial infection may depend upon the manner of administration, the age and the body weight of the subject and the condition of the subject to be treated. Generally, the antibacterial agent is administered to a subject at a dosage of 1 to 100 mg/kg of body weight (preferably at a dosage of 1 to 10 mg/kg of body weight).

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#### Use of Inhibitors of Bacterial Virulence

FAB inhibitors that also are inhibitors of HSL synthesis can be used to inhibit the expression of bacterial virulence factors. As described above for FAB inhibitors generally, inhibitors of bacterial virulence can be administered to an organism in need of treatment.

10 Like other FAB inhibitors identified with the methods of the invention, inhibitors of bacterial virulence can be used to treat infections caused by a broad spectrum of bacteria.

#### EXAMPLE

15 The invention is further described by the following example, which is not intended to limit the scope of the invention.

#### Identification of the PyhfB, PyjaXY, PylpC, and PyydK Promoters

Conventional RNA profiling studies revealed that bacterial cells treated with the FAB pathway inhibitors cerulenin (0.5 µg/ml or 5 µg/ml) and triclosan (0.1 µg/ml or 1 µg/ml) in 20 LB medium at 37°C for 10, 30, or 60 minute time periods displayed an increase in activity of the PyhfB, PyjaXY, PylpC, and PyydK promoters. Thus, transcriptional profiling indicated that the promoters of these genes were upregulated by antibacterial agents.

25 *B. subtilis* strains containing a PyhfB, PyjaXY, or PylpC promoter (along with an insignificant portion of the coding sequence) operably linked to the coding sequence of an *E. coli lacZ* reporter gene were used to confirm that the activity of the promoter is upregulated by an inhibitor of fatty acid biosynthesis. Clones containing the PyhfB, PyjaXY, and PylpC promoters have been described in GenBank, and the accession numbers for these clones are set forth in Table 2. The promoters were isolated by PCR amplification of *B. subtilis* genomic DNA using the primers shown in Table 2.

30 The reporter strains used in these assays were constructed as follows. PCR products containing the promoters were digested with *EcoRI* and *BamHI*, and cloned into the multiple cloning site of pDG268, which also had been digested with *EcoRI* and *BamHI*. The plasmid

pDG268 is a derivative of pGD364 (See, Cutting and Van der Horn, *Molecular Biological Methods for Bacillus*, eds. C.R. Harwood and S.M. Cutting, 1990, John Wiley and Sons, NY, NY, pp. 52-54). A schematic representation of pDG268 is set forth in Fig. 6. The plasmid pDG268 is able to replicate in *E. coli*, contains an ampicillin-resistance gene, and encodes a promoter-less *lacZ* coding sequence. The multiple cloning site (MCS) is positioned such that a promoter of interest can be directionally cloned to drive expression of the *lacZ* sequence. Adjacent to the MCS, and opposite the *lacZ* sequence, is a gene encoding chloramphenicol (Cm) resistance for *B. subtilis*. The entire Cm-MCS-*lacZ* region is flanked by the upstream and downstream section of the *B. subtilis amyE* gene (a "silent" locus in the *B. subtilis* chromosome). Thus, the promoter fusion of interest can be stably integrated by homologous recombination at the *amyE* site of the *B. subtilis* chromosome by transforming *B. subtilis* cells with the linearized plasmid, and selecting for resistance to chloramphenicol. The promoter fusions were placed at the *amyE* chromosomal location of the *B. subtilis* strain PY79, which is a wild-type *B. subtilis* strain that is devoid of prophages (Youngman et al., 1984, *Mol. Gen. Genet.* 195:424-433). The resulting strains were: PY79(*amy::PyhfB::lacZ. cat*), PY79(*amy::PylpC::lacZ. cat*), PY79(*amy::PydK::lacZ. cat*), and PY79(*amy::PyjaXY::lacZ. cat*).

**Table 2: Primers Used to Isolate Promoters**

| Gene         | GenBank Accession No. | Upstream Primer  | Downstream Primer                               |
|--------------|-----------------------|--|---|
| <i>yhfB</i>  | BG13048               | 5' TTTTCTTTTTCATA<br>GATTCCTATCTACA3'<br>(SEQ ID NO:5) | 5' AAGCGTTCCTTT<br>ATAACGGC3'<br>(SEQ ID NO:6)  |
| <i>yjaXI</i> | BG13127               | 5' CACATCCAACCTGC<br>ATACGCC3'<br>(SEQ ID NO:7)        | 5' ATTCAATAAATT<br>GTTTACCG3'<br>(SEQ ID NO:8)  |
| <i>ylpC</i>  | BG13400               | 5' TGTGTTAAGAGAT<br>GAATTGC3'<br>(SEQ ID NO:9)         | 5' AACTTCATCAAG<br>TGACAGGG3'<br>(SEQ ID NO:10) |
| <i>yvdK</i>  | BG11484               | 5' CTAGTCTCGTATA<br>ATCTCTC3'<br>(SEQ ID NO:11)        | 5' AGGTGACGTTCA<br>AATCCTCG3'<br>(SEQ ID NO:12) |

The reporter strains were incubated, separately, with triclosan or cerulenin at various concentrations, as shown on the X-axes depicted in Figs. 7 and 8. Each strain and an

antibacterial agent were incubated in a 96-well microplate for 3 hours.  $\beta$ -galactosidase activity was detected using the Tropix Galacton Star<sup>TM</sup> chemiluminescent substrate (3-chloro-5-(4-methoxyspiro{1.2-dioxetane-3.2'-(4'chloro)-tricyclo-[3.3.1.1<sup>3,7</sup>] decan}-4-yl)phenyl-B-D-galactopyranoside) and the detection method described below. The fold induction of promoter activity was calculated as the level of activity in the samples containing the antibacterial agent divided by the level of activity in the samples that lacked the agent. The results obtained with the strain PY79(*amy::PyhfB::lacZ, cat*) are represented by Fig. 7, and the results obtained with the strain PY79(*amy::PylpC::lacZ, cat*) are represented by Fig. 8.

The minimum inhibitory concentrations (MIC) for cerulenin and triclosan were determined for each of the reporter strains. To determine the MIC, a compound of interest (e.g., cerulenin or triclosan) was placed in a 96-well microtiter plate such that the concentration of the compound varies in various wells of the plate (e.g., by using a series of 2-fold dilutions of the compound). Approximately 5,000 cells from a logarithmically-growing culture (e.g., the *B. subtilis* strain PY79) in Luria broth were added to each well (e.g., in a volume of 50-80  $\mu$ l). The plate then was incubated at 37°C for approximately 18 hours. The OD<sub>600</sub> was then read to measure cell growth in each well of the plate. The lowest concentration of the compound that leads to complete killing of the culture (i.e., the concentration of compound for which the OD<sub>600</sub> nm is equal to a control well that contains only media and the test compound) is deemed the MIC for the particular compound and bacterial strain. The MICs for cerulenin and triclosan were 12.5  $\mu$ g/ml and 5  $\mu$ g/ml, respectively, for both of the reporter strains. Such inhibitors can be used as lead compounds, which can subsequently be derivatized using standard medicinal chemistry techniques to produce antibacterial agents. The PY79(*amy::PyjaXY::lacZ, cat*) strain did not provide significant induction of promoter activity.

#### Assay for Inhibitors of FAB

Having determined that antibacterial agents such as cerulenin and triclosan increase the activity of the PyhfB and PylpC promoters, bacterial strains containing one or more of these promoters each operably linked to a reporter gene (e.g., *lacZ*) can be used in a method for determining whether a test compound is an inhibitor of FAB. The following example of such a method describes use of the PyhfB promoter in an initial assay and use of the PylpC



promoter in an optional confirmation assay. Comparable reagents, apparatuses, and methods can readily be substituted for those described herein.

Bacterial Strains: The bacterial strain PY79 (*amy::PyhfB::lacZ, cat*), which contains the PyhfB promoter operably linked to the coding sequence of a *lacZ* reporter gene, was used in the initial screen for inhibitors of FAB. To prepare a bacterial glycerol stock, the cell culture was mixed at a 1:1 ratio with sterile 50% glycerol, and 1.0 ml aliquots of the suspension were stored in cryovials at -80°C.

To prepare cells for use in the assay of test compounds, 5 µl of the glycerol stock was diluted in 80 ml of low salt LB medium (LB broth containing 5 g/L of NaCl). A 15 µl aliquot of this diluted glycerol stock was then added to 900 ml of low salt LB medium and grown to an OD<sub>600</sub> of 0.6 in a shaking incubator at 30°C. Generally, a culture having an OD<sub>600</sub> of 0.4 to 0.6 is suitable for use in the screening assays.

Screening Plates: In this example, chemical libraries containing test compounds at 10 mg/ml in DMSO are diluted to 100 µg/ml with 50 mM HEPES buffer (pH 7.4). The test compounds (e.g., 0.1 µg or 0.05 µg) are dispensed into each well of a microplate (e.g., a 96- or 384-well microplate). The dispensed compounds are dried onto the plates (e.g., by leaving the plates in a fume hood overnight), and the plates are stored in the freezer and then brought to room temperature before being used in the screening assay.

A 50 µl sample of the bacteria is added to each well of the microplate. Cerulenin is used as a control in wells of the microplate that lack a test compound. A stock solution of cerulenin (2,3-Epoxy-4-oxo-7,11-dodecadienamide) is prepared to a concentration of 10 mg/ml in DMSO and stored at -20°C. A working solution of cerulenin is prepared by diluting the cerulenin stock solution to 50 µg/ml with 50 mM HEPES (pH 7.4). A 5 µl aliquot of the 50 µg/ml of cerulenin working solution is added to the plate to provide a control. The bacteria and test compounds (or cerulenin control) are then incubated at 30°C for approximately 5 hours. If desired, the length and/or temperature of incubation can be increased or decreased. After incubating the cells and test compounds, the plates can be stored at -80°C if they are not going to be analyzed immediately. Before analysis, the plates should be returned to room temperature, e.g., by leaving the plates in a single layer for one hour at room temperature.

Assay for Induction of Promoter Activity: To assay for an increase in the activity of the PyhfB promoter, an increase in expression of the *lacZ* reporter gene expression (or expression of an alternative reporter gene) is measured. A 50 µl aliquot of 2X substrate buffer is added to each well of the plate. To produce a 10 ml sample of 2X substrate buffer, the following components are mixed and kept at room temperature: 0.4 ml of Galacton-Star™ substrate (3-chloro-5-(4-methoxyspiro{1.2-dioxetane-3.2'-(4'chloro)-tricyclo-[3.3.1.1<sup>3,7</sup>] decan}-4-yl)phenyl-B-D-galactopyranoside; Tropix, Inc., Cat.# GS100); 2.0 ml of Sapphire II™ luminescence signal enhancer (Tropix, Inc., Cat.#LAX250); and 7.6 ml of lysis buffer (0.026% Na Deoxycholic acid, 0.053% CTAB, 265 mM NaCl, 395 mM HEPES, pH 7.5). The 2X substrate buffer is stable for approximately 2 hours. The plates are incubated at room temperature for 120 minutes before measuring β-galactosidase activity.

A chemiluminescent signal is produced by reaction of β-galactosidase with the Galacton-Star™ substrate (3-chloro-5-(4-methoxyspiro{1.2-dioxetane-3.2'-(4'chloro)-tricyclo-[3.3.1.1<sup>3,7</sup>] decan}-4-yl)phenyl-B-D-galactopyranoside). Chemiluminescence is measured using a TopCount™ microplate reader set as follows: data mode = seconds per count; count time = 0.1 minute; count delay = 0.10 minute; background subtract = none; half-life correction = no; sample screening = no; and quench indicator = tSIS. Generally, chemiluminescence should be measured before significant signal decay occurs. The read window for the substrate, during which time the plates should be analyzed, is approximately 120 minutes to 180 minutes after initiating the reaction.

The mean and standard deviation of the results obtained for the reactions on a particular plate are measured (excepting the cerulenin control wells). A chemiluminescent signal that is at least 3 times the standard deviation, plus the mean, indicates that the test compound is a "lead" compound, i.e., a compound that increases the activity of the promoter and is a FAB inhibitor. The cerulenin controls described above should produce a chemiluminescent signal that is at least equal to 4 times the mean. The general quality of the analysis of the plate can be determined using Spotfire Pro version 4.0 data analysis software (Spotfire, Inc.: Cambridge, MA) by assessing parameters such as the overall hit rate, the random nature of the position of hits, and signal strength consistency over all plates and analyses. Compounds that inhibit FAB may or may not be bacteriocidal or bacteriostatic. Nevertheless, such compounds can be used in the development of antibacterial drugs through the use of standard medicinal chemistry techniques. Thus, the invention includes methods

for preparing an antibacterial agent by: screening multiple test compounds as described herein to identify candidate compounds that upregulate promoter activity and therefore are FAB inhibitors; isolating one or more lead compounds from the candidate compounds; determining whether the compound inhibits the growth of a bacterium, in which case the compound can be formulated as an antibacterial agent; and, optionally, derivatizing the lead compound(s) through medicinal chemistry to produce a derivative(s) that inhibits the growth of a bacterium; and formulating the derivative as an antibacterial agent. Optionally, the compound can be characterized in a biochemical assay to determine which step it inhibits in the FAB pathway and/or confirm that the protein is a FAB inhibitor.

Confirmatory Assay: Test compounds that cause an induction of promoter activity of at least 3 times the standard deviation, plus the mean, can be further tested (e.g., at 10 µg/ml and 50 µg/ml) in an optional, "confirmatory" assay. This assay is carried out identically to the above-described assay, except that a bacterial strain containing an alternate promoter operably linked to a reporter gene is used. For example, if the strain PY79(*amy::PyhfB-lacZ, cat*) is used in the initial screening assay, the strain PY79(*amy::PylpC-lacZ, cat*) can be used in the confirmatory assay. Other combinations of promoters also can be used in the initial and confirmatory assays. Of course, the test compounds can be re-tested with the strain used in the initial screen. Because the test compounds used in such a confirmatory assay can be expected to result in a high overall level of induction, the wells in four columns of the microplate are used as controls (lacking the test compound). The mean and standard deviation are determined using the values obtained with these wells (minus the cerulenin controls).

Biochemical Assay of Inhibition of Fatty Acid Biosynthesis: If desired, test compounds identified as antibacterial agents in the above-described assays can be further tested for their ability to inhibit fatty acid biosynthesis in a biochemical assay. For example, compounds identified in the above-described assay can be further tested for their ability to inhibit the enzymes encoded by fatty acid biosynthesis genes, such as *fabD*, *fabH*, *fabG*, *fabA*, *fabI*, *fabF/B*, *yjaX*, *yhfB*, *ywpB*, *yjbW*, or *yjaY*. The reactions catalyzed by each of these gene products are set forth above in Table 1, and methods for assaying these reactions are known in the art.

Other Embodiments

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other  
5 aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1           1. A method for determining whether a test compound is an inhibitor of bacterial  
2 fatty acid biosynthesis (FAB), the method comprising:  
3           (i) contacting a bacterial cell with a test compound, wherein the bacterial cell  
4 contains  
5           (a) a promoter, the activity of which is increased in the presence of a compound  
6 that inhibits FAB, operably linked to  
7           (b) a reporter gene; and  
8           (ii) measuring activity of the promoter, wherein an increase in activity, relative to the  
9 level of activity of the promoter in the absence of the test compound, indicates that the test  
10 compound is an inhibitor of bacterial FAB.

1           2. A method of claim 1, wherein the reporter gene is selected from the group  
2 consisting of *lacZ*, *cat*, *gus*, a luciferase gene, and a green fluorescent protein gene.

1           3. A method of claim 1, wherein measuring an increase in activity of the promoter  
2 comprises measuring binding of antibodies to a product of the reporter gene.

1           4. A method of claim 1, wherein the promoter is *PyhfB* or *PylpC*.

1           5. A method for determining whether a test compound is an antibacterial agent, the  
2 method comprising:  
3           (i) contacting a bacterial cell with a test compound, wherein the bacterial cell  
4 contains  
5           (a) a promoter, the activity of which is increased in the presence of a compound  
6 that inhibits fatty acid biosynthesis (FAB), operably linked to  
7           (b) a reporter gene;  
8           (ii) measuring activity of the promoter, wherein an increase in activity, relative to the  
9 level of activity of the promoter in the absence of the test compound, indicates that the test  
10 compound is an inhibitor of bacterial FAB; and  
11           (iii) determining whether the inhibitor of FAB inhibits the growth of a bacterium,  
12 wherein a compound that inhibits the growth of a bacterium is an antibacterial agent.

1           6. A method of claim 5, further comprising assaying the test compound for its ability  
2   to inhibit FAB.

1           7. A method of claim 6, wherein inhibition of FAB is detected as inhibition of  
2   incorporation of acetate into a fatty acid or phospholipid.

1           8. A method of claim 7, wherein inhibition is measured in an extract of the cell.

1           9. A method of claim 5, wherein the promoter is PyhfB or PylpC.

1           10. A method for treating a bacterial infection in an organism, the method  
2   comprising administering an effective amount of an antibacterial agent identified by the  
3   method of claim 5 to an organism having a bacterial infection, thereby treating the bacterial  
4   infection in the organism.

1           11. A method of claim 10, wherein the organism is a mammal.

1           12. A method of claim 11, wherein the mammal is a human.

1           13. A method of claim 10, wherein the bacterial infection is caused by *Streptococcus*  
2   *pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus endocarditis*,  
3   *Streptococcus faecium*, *Streptococcus sanguis*, *Streptococcus viridans*, or *Streptococcus*  
4   *hemolyticus*.

1           14. A method of claim 10, wherein the bacterial infection is an infection by a  
2   pathogenic bacterium.

1           15. A method of claim 10, wherein the bacterial infection is an infection by a non-  
2   pathogenic bacterium.

1           16. A composition comprising an antibacterial agent identified by the method of  
2   claim 5 and a pharmaceutically acceptable carrier.

1 17. A method of preparing an inhibitor of bacterial FAB, the method comprising:  
2 screening multiple test compounds by the method of claim 1;  
3 identifying candidate compounds that upregulate promoter activity;  
4 isolating one or more lead compounds from the candidate compounds;  
5 identifying and selecting a lead compound that inhibits bacterial FAB; and  
6 formulating the selected lead compound as an inhibitor of bacterial FAB.

1 18. A method of preparing an antibacterial agent, the method comprising:  
2 screening multiple test compounds by the method of claim 5;  
3 identifying candidate compounds that upregulate promoter activity;  
4 isolating one or more lead compounds from the candidate compounds;  
5 identifying and selecting a lead compound that inhibits growth of a bacterium; and  
6 formulating the selected lead compound as an antibacterial agent.

1 19. A method for preparing an inhibitor of bacterial FAB, the method comprising:  
2 screening multiple test compounds by the method of claim 1;  
3 identifying candidate compounds that upregulate promoter activity;  
4 isolating one or more lead compounds from the candidate compounds;  
5 derivatizing said lead compound(s), thereby producing derivatives of said lead  
6 compound;  
7 identifying one or more derivatives that inhibit bacterial FAB; and  
8 formulating one or more derivatives as inhibitors of bacterial FAB.

1 20. An inhibitor of bacterial FAB prepared by the method of claim 19.

1 21. A method for inhibiting bacterial fatty acid biosynthesis in bacteria infecting an  
2 organism, the method comprising administering an effective amount of an inhibitor of  
3 claim 20 to an organism having a bacterial infection, thereby inhibiting bacterial fatty acid  
4 biosynthesis in the bacteria.

1 22. A method for preparing an antibacterial agent, the method comprising:  
2 screening multiple test compounds by the method of claim 5;

3 identifying candidate compounds that upregulate promoter activity;  
4 isolating one or more lead compounds from the candidate compounds;  
5 derivatizing said lead compound(s), thereby producing derivatives of said lead  
6 compound:  
7 identifying one or more derivatives that inhibit growth of a bacterium: and  
8 formulating one or more derivatives as an antibacterial agent.

1 23. An antibacterial agent prepared by the method of claim 22.

1 24. A composition comprising an antibacterial agent of claim 23 and a  
2 pharmaceutically acceptable carrier.

1 25. A method for inhibiting growth of bacteria in an organism having a bacterial  
2 infection, the method comprising administering an effective amount of a composition of  
3 claim 24 to an organism having a bacterial infection, thereby inhibiting growth of bacteria in  
4 the organism.

1 26. A method for determining whether a test compound is an inhibitor of bacterial  
2 virulence, the method comprising:

3 (i) contacting a bacterial cell with a test compound, wherein the bacterial cell  
4 contains

5 (a) a promoter, the activity of which is increased in the presence of a compound  
6 that inhibits FAB, operably linked to

7 (b) a reporter gene;

8 (ii) measuring activity of the promoter, wherein an increase in activity, relative to the  
9 level of activity of the promoter in the absence of the test compound, indicates that the test  
10 compound is an inhibitor of bacterial FAB; and

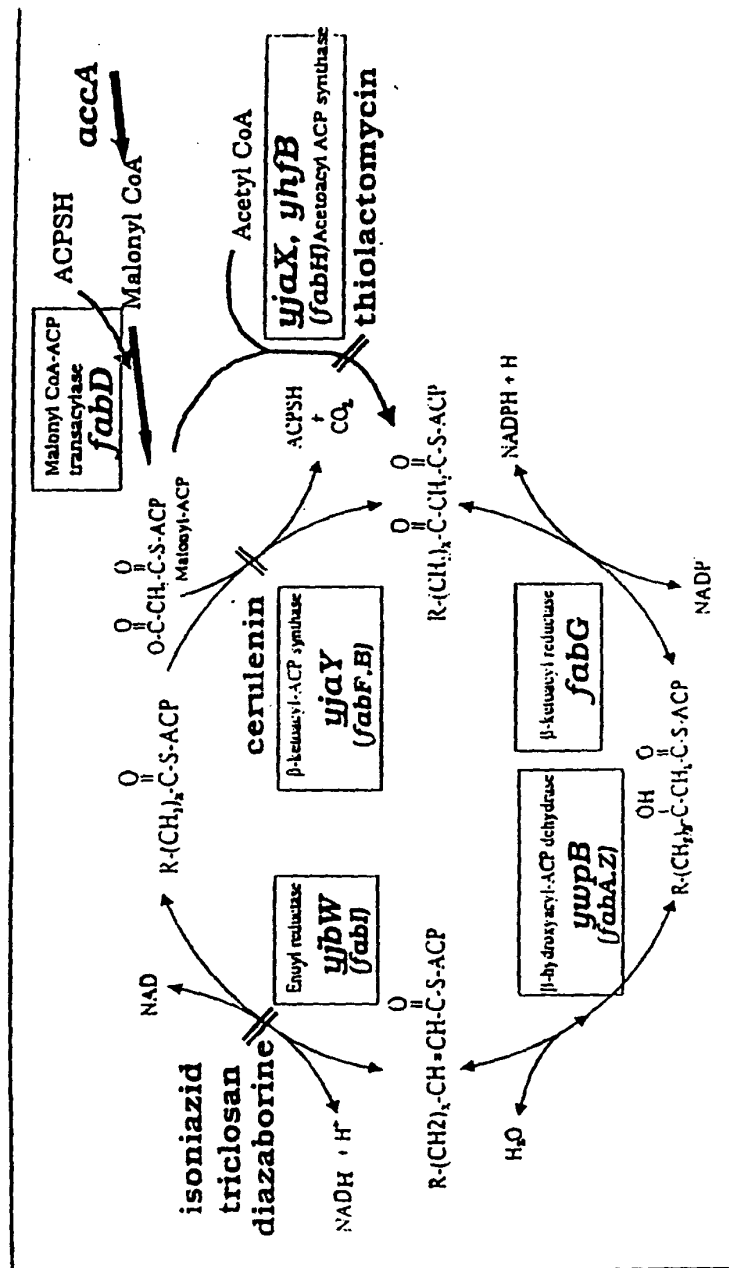
11 (iii) determining whether the inhibitor of FAB inhibits acylated homoserine lactone  
12 synthesis, wherein a compound that inhibits acylated homoserine lactone synthesis is an  
13 inhibitor of bacterial virulence.



- 1           27. A method of claim 26, wherein inhibition of acylated homoserine lactone  
2           synthesis is detected as inhibition of enoyl-acyl carrier protein reductase activity.
- 1           28. A method of claim 26, wherein the promoter is Pyhfb or PylpC.
- 1           29. A method for inhibiting bacterial virulence in bacteria infecting an organism, the  
2           method comprising administering an effective amount of an inhibitor of bacterial virulence  
3           identified by the method of claim 26 to an organism having a bacterial infection, thereby  
4           inhibiting virulence in the bacteria infecting the organism.
- 1           30. A method of claim 29, wherein the organism is a mammal.
- 1           31. A method of claim 30, wherein the mammal is a human.
- 1           32. A method of claim 29, wherein the bacterial infection is caused by *Streptococcus*  
2           *pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus endocarditis*,  
3           *Streptococcus faecium*, *Streptococcus sanguis*, *Streptococcus viridans*, or *Streptococcus*  
4           *hemolyticus*.
- 1           33. A method of claim 29, wherein the bacterial infection is an infection by a  
2           pathogenic bacterium.
- 1           34. A composition comprising an inhibitor of bacterial virulence identified by the  
2           method of claim 26 and a pharmaceutically acceptable carrier.
- 1           35. A method of preparing an inhibitor of bacterial virulence, the method  
2           comprising:  
3           screening multiple test compounds by the method of claim 26;  
4           identifying candidate compounds that inhibit acylated homoserine lactone synthesis;  
5           isolating one or more lead compounds from the candidate compounds;  
6           identifying and selecting a lead compound that inhibits bacterial virulence; and  
7           formulating the selected lead compound as an inhibitor of bacterial virulence.

- 1           36. A method for preparing an inhibitor of bacterial virulence, the method  
2 comprising:  
3           screening multiple test compounds by the method of claim 26;  
4           identifying candidate compounds that inhibit acylated homoserine lactone synthesis;  
5           isolating one or more lead compounds from the candidate compounds;  
6           derivatizing said lead compound(s), thereby producing derivatives of said lead  
7 compound;  
8           identifying one or more derivatives that inhibit bacterial virulence; and  
9           formulating one or more derivatives as an inhibitor of bacterial virulence.
- 1           37. An inhibitor of bacterial virulence prepared by the method of claim 35.
- 1           38. A composition comprising an inhibitor of bacterial virulence of claim 37 and a  
2 pharmaceutically acceptable carrier.
- 1           39. A method for inhibiting bacterial virulence in an organism having a bacterial  
2 infection, the method comprising administering an effective amount of the composition of  
3 claim 38 to an organism having a bacterial infection, thereby inhibiting bacterial virulence in  
4 the organism.

**FIG. 1**



**FIG. 2**

## PyhfB Promoter Sequence

(SEQ ID NO: 1)

```
TTTTCTTTTCATAGATTCCTATCTACActtctctttttttgtaaaaaacc  
ttcttttttagtaccagataactaatatctagtgttataatctgaccataagg  
agtgattcatatgtcaaaagcaaaaattacagctatcggcacctatgcgcc  
gagcagacgtttaaccaatgcagatttagaaaaagatcgttgatacctctga  
tgaatggatcggttcagcgcacaggaatgagagaacgccggattgcggatga  
acatcaatttacctctgatttatgcatagaagcgggtgaagaatctcaagaG  
CCGTTATAAAGGAACGCTT
```

**FIG. 3****PyjaXY Promoter Sequence**

(SEQ ID NO:2)

CACATCCAAC TGCATACGCC Tcctttccatataccatactctatgagtaag  
atgaactgatagtttagacgaatatattgccatgtgaaaaaaaaataggata  
gaattagtagctgatactaataattgatcacacctgattgatcttctaaa  
tttaagatataaaaggagtcttccctaataagaaagctggaataacttggtgttg  
gacgttacattcctgagaagggtttaacaaatcatgatcttgaaaaaatgg  
ttgaaacttctgacgagtggattcgtacaagaacaggaatagaagaaagaa  
gaatcgcagcagatgatgtgttttcatcacatatggctggtgcagcagcga  
aaaatgcgctggaacaagctgaagtggctgctgaggatctggatatgatct  
tggttgcaactgttacacctgatcagtcattccctacggtctcttgatatga  
ttcaagaacaactcggcgcggaagaaagcgtgtgctatggatatcagcgcg  
cttgtgcgggcttcatgtacggggttgtaacCGGTAAACAATTTATTGAAT

**FIG. 4****PylpC Promoter Sequence**

(SEQ. ID NO:3)

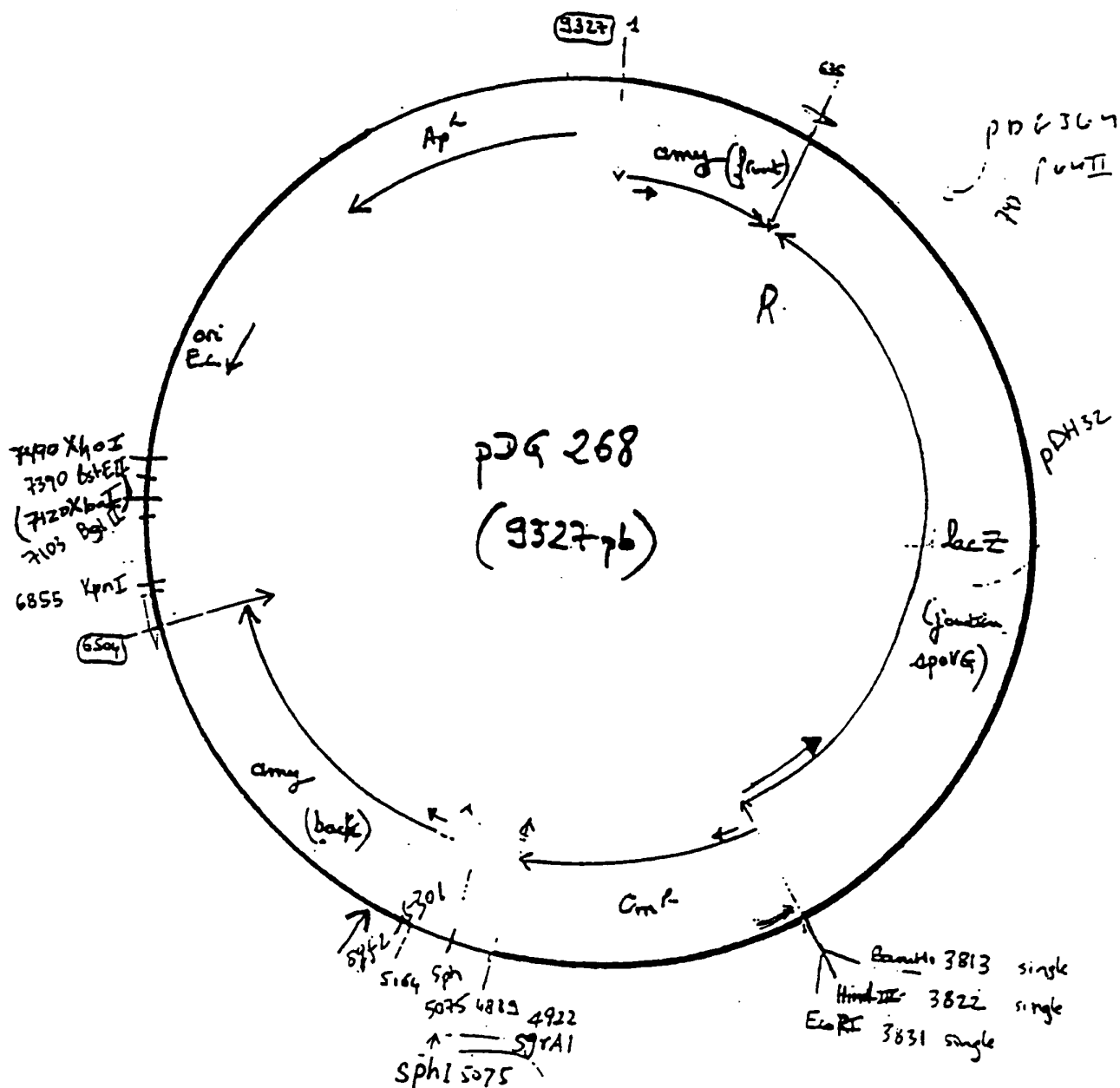
TGTGTTAAGAGATGAATTGCTgaagagcggagtaatggacggggaaaaatt  
aagctgagtatcagaagtttttggtgttcagccagaaacaatcgttgcatt  
ccaatctatgaaattatatactactattagtagtcttaattgtccgg  
atggtgttttagatatgagaagaaataagagagaacgccaggaattacttca  
gcagacgattcaagcaacccccctttattacagatgaagaactagcgggtaa  
attcggggtgagcatccagacgatacgtttggaccgcttagagctttccat  
acctgaactgagagaaagaattaagaacgtggcagagaaaacacttgagga  
cgaagtgaagtCCCTGTCACTTGATGAAGTT

**FIG. 5****PyydK Promoter Sequence**

(SEQ ID NO:4)

CTAGTCTCGTATAATCTCTCactttcccccgcacatttcacccaccccttcgt  
tttgggttacgctttcattataattgtaacgggtataatztatcaattccgaa  
acaaaatattttacgcactaactatcattgtaagcggtttatgctataat  
tgacaagtgaactaaacgaataaatgaggaatgcgtatgttaaaatacca  
gcaaatcgcaacggaaattgaaacatatatagaagaacaccagcttcagca  
gggagacaaaactgccagtcctagaaaccctcatggcccagtttgaagtcag  
caaaagcaccatcacaaagtccctcgagctattagagcaaaaaggcgcgat  
ctttcaggtcagaggaagcggcattttcgtcagaaaacataaacgaaaagg  
ctatatcagccttctgtcgaatcagggatataaaaaagatctCGAGGATT  
CAACGTCACCT

FIG. 6

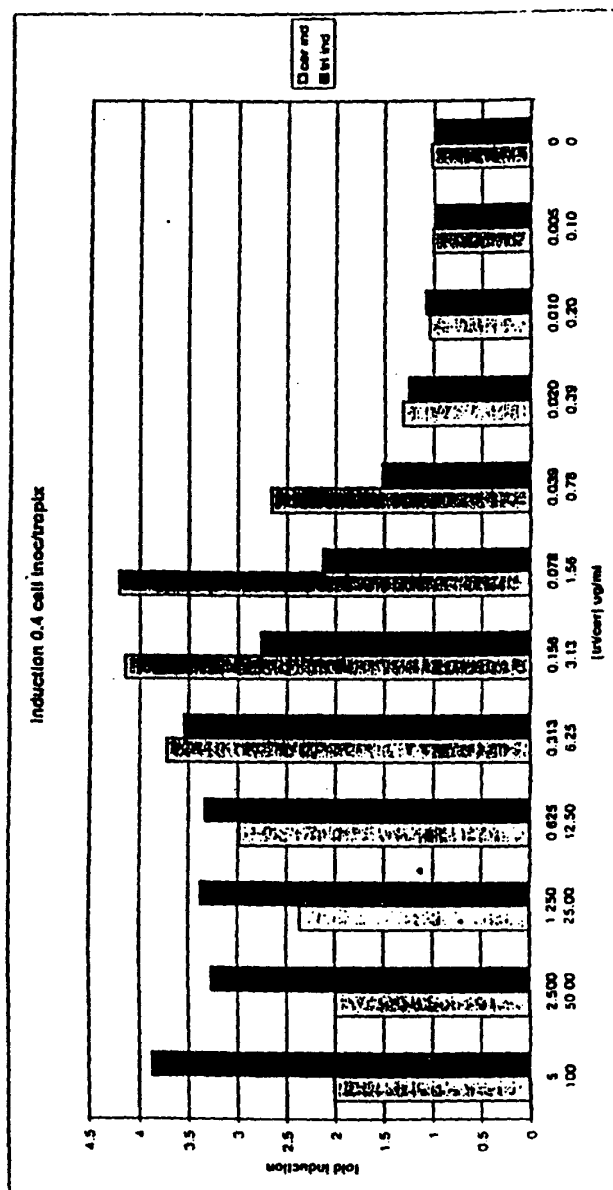




20/089019

FIG. 7

yhfB::lacZ strain results



## yIpC::lacZ strain results

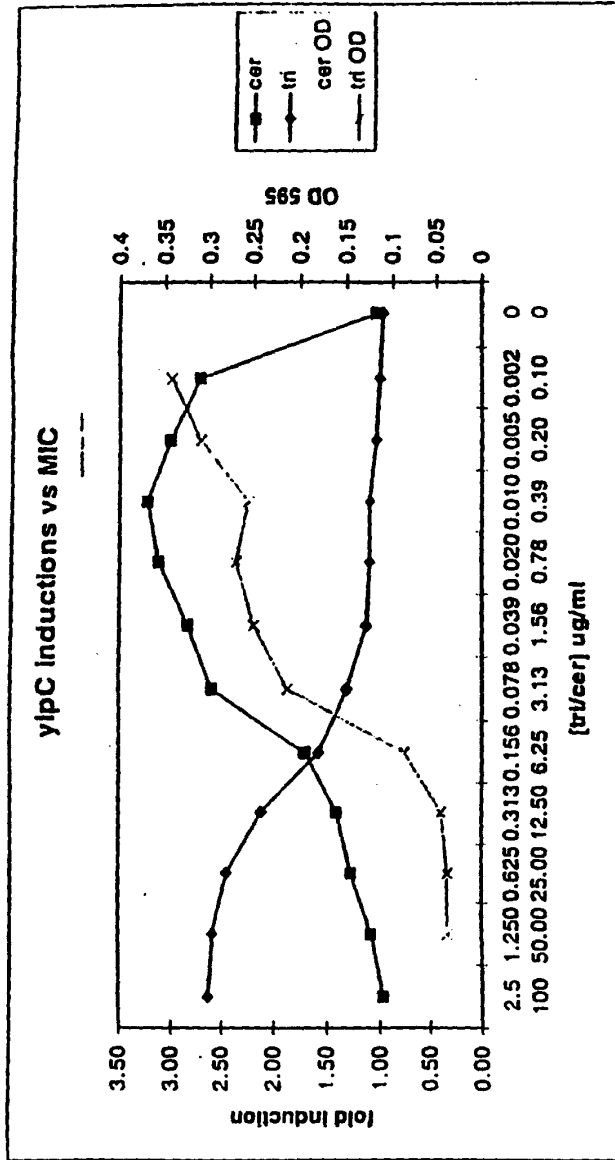


FIG. 8